John H. Andrews
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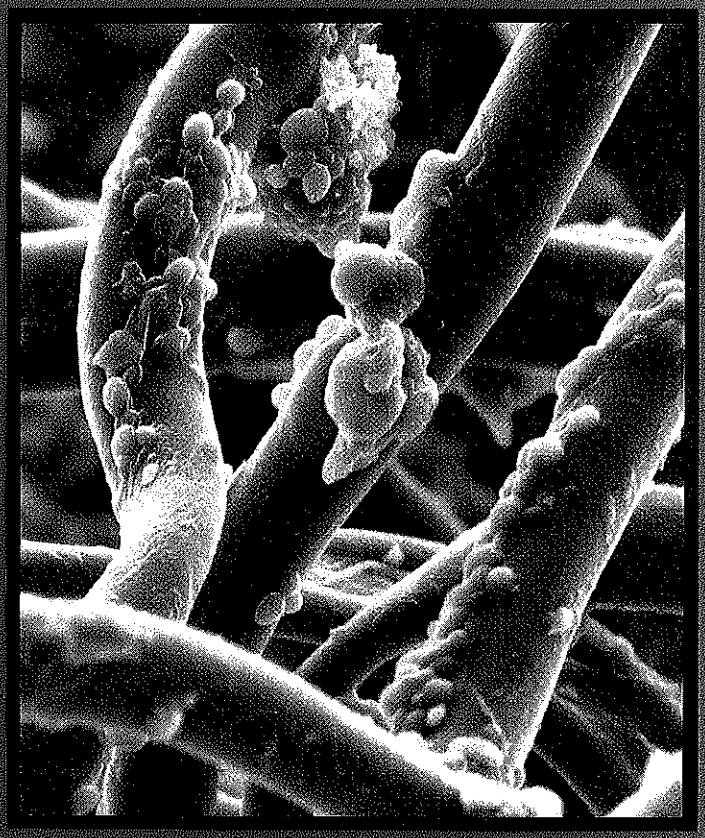
Microbial Ecology of Leaves



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The phyllosphere or leaf is a major habitat for microorganisms. Microbes on or within leaves play important roles in plant ecology and these microbes can be manipulated to enhance plant growth or reduce plant disease. This book presents a number of critical reviews by internationally recognized experts on the microbial ecology of leaves. Topics include methods of assessment of microbial populations on leaf surfaces, leaves as reservoirs of medically important microbes, the evolutionary biology of leaf microbes, the ice nucleation phenomenon, and leaves as microbial habitats in both aquatic and terrestrial environments. This book will be of interest to students and scientists in numerous disciplines, including botany, aerobiology, meteorology, ecology, agriculture, microbiology, and biotechnology.

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Genetically Engineered Endophytes as Biocontrol Agents: A Case Study from Industry

Jed W. Fahey, Michael B. Dimock, Steven F. Tomasino, Jean M. Taylor, and Peter S. Carlson

20.1 Introduction

Economic, social, and political forces determine which new technologies are utilized. Commercial incentives and environmental standards are the driving forces underlying the development and application of biotechnology. Agricultural biotechnology's customer, the farmer, is a price- and risk-sensitive consumer. Inexpensive, proven technologies that demand little or no change in agronomic practices are, under ordinary conditions, preferred by farmers. Growers are reluctant to increase the portion of their costs devoted to planting materials at the beginning of the growing season. Presently, biotechnology is neither an inexpensive nor a proven method for increased crop production, and its application may alter agronomic techniques. Until proven otherwise, the case can be made that the added value created by many biotechnological manipulations may not be adequately reimbursed by the market.

The agricultural technologies currently used in the developed world produce sufficient foodstuffs. The use of biotechnology to increase food output may not be as readily accepted as the use of biotechnology to redress the negative environmental and ecological impacts of current agricultural practices.

20.2 Endophytes for Crop Protection

Although numerous plant-endophyte interactions have been identified over the past century, only a few species of endophytic microorganisms

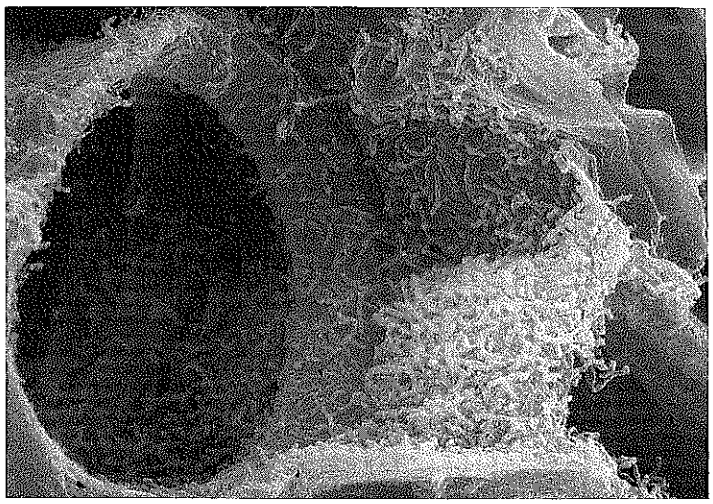

Figure 20.1 Lumen of a xylem vessel from the stem of 4-week-old *Zea mays* var. FR632 colonized with endophytic bacterium *Clavibacter xyli* subsp. *cynodontis*. The freeze-fracture surface reveals annular rings on the left side of the photograph. (Approximate magnification, ×3,500.)

(e.g., *Rhizobium*) have been used commercially for their agricultural potential as symbionts. Genetic engineering of nonpathogenic endophytic bacteria presents an opportunity for the systemic delivery of biopesticides (fungicides and insecticides) within host plant tissues without direct genetic manipulation of the host plant. The advantages of such a strategy include sustained and protected activity of the inhibitory compound.

Such products could be delivered as single-application seed treatments. This approach exploits the biological characteristics of a natural endophytic microbe, which can systemically colonize the xylem (Figure 20.1), and persist in host plants. Because of the containment of the biocontrol agent within the plant's vascular system and its inability to survive when the host dies, effects on nontarget organisms and the environment should be reduced. Thus, the use of endophytes should have significant economic, environmental, and technological advantages over the use of current externally applied agrichemicals. Xylem-inhabiting bacteria are not ubiquitous, but certain species can be recovered from plants in a pattern which suggests that they may be part of the host's normal microflora. For example, bacteria from 13 different genera have been isolated from the xylem of healthy *Citrus* trees at levels of up to 2×10^4 CFU/g of xylem (Gardner et al., 1982; R.M. Zablotowicz, personal communication). In addition, several different bacterial isolates, including species of *Erwinia* and *Bacillus*, have been recovered from symptom-free cotton plants, and they were shown to colonize systemically following inoculation (Misaghi and Donndelinger, 1990). Furthermore, xylem-inhabiting endophytes may be responsible for the fre-

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quent presence of microflora in surface-disinfested explant material. The failure of excised tissues of certain plant species to grow in vitro could in fact be related to a stimulatory effect of an endophytic microbe that cannot be reproduced by culturing isolated, bacteria-free explants.

Methods have been developed by the authors and others at Crop Genetics International (CGI), Hanover, MD, to screen, identify, recover, and characterize endophytic microorganisms. We have conducted an extensive search for endophytes capable of colonizing the major crop species and have developed a collection of putative endophytes. Microorganisms are selected for this collection based on their ability to live inside and inability to survive outside the target crops.

Quantitative isolations from asymptomatic target plant species following rigorous surface disinfestation procedures were used to initially isolate and further characterize bacteria from systemic populations. One such microbe is *Clavibacter xyli* subsp. *cynodontis* (Cxc), a fastidious, Gram-positive, coryneform bacterium. CGI has used Cxc in the first of a planned series of InCide™ biopesticide products. To date, Cxc has only been found to occur naturally in the xylem of bermudagrass (*Cynodon dactylon*) stems, leaves, and roots; it is usually the exclusive occupant of this niche. Species of bacteria that are presently classified as *Clavibacter* were previously assigned to the genus *Corynebacterium* (Davis et al., 1984).

Creating novel crop varieties through biotechnology is generally not a business with an attractive return on investment. No matter how carefully crafted the patent protection may be, the farmer's field is, in effect, a genetic photocopier. The one-time sale of a biotechnology-based new variety cannot support a price structure adequate to cover research costs. A "repeat-sales" mechanism such as hybridization is needed for commercialization. Protecting crops from within via genetically engineered endophytes represents a unique market niche, and InCide biopesticides based on Cxc create their own "repeat-sales" opportunity because they are not seed transmitted.

The first InCide product involves a genetically engineered Cxc capable of producing the delta endotoxin of *Bacillus thuringiensis* subsp. *kurstaki* (Cxc/Bt), a protein that is toxic to the larvae of many species of Lepidoptera (caterpillars). This product can be inoculated into corn to control the European corn borer (ECB) *Ostrinia nubilalis*. There is little published information on Cxc. Thus, CGI has generated extensive data (Experimental Use Permit applications submitted to the U.S. EPA in 1987–1990; see EUP58788-EUP1 1987; -EUP2, 1989; -EUP4, 1990) on the biology, ecology, pathology, and environmental fate of this organism in order to aid in product development and registration.

Cxc has proven to be widespread within the geographic range of its natural host, bermudagrass, based on extensive sampling of bermudagrass populations over the past five years. Cxc has been isolated from bermudagrass in Taiwan, France, Japan, and 26 states within the U.S., including the

leading corn-producing states such as Nebraska, Illinois, and Indiana (Chen et al., 1977; EUP58788-EUP1).

Once introduced into corn seedlings by wound inoculation of the stems of young plants, or by seed inoculation, Cxc colonizes the xylem of roots, stems, leaves, and husks. Cxc populations of over 10^9 bacteria per gram of fresh tissue can be achieved within 2–4 weeks of planting inoculated seed. Cxc has been inoculated into over 100 commercial hybrids, all of which were colonized, generally in the range of 10^5–10^9 CFU/g fresh tissue. Since it is a vascular-limited endophyte, Cxc is not present in the seed of colonized plants. Host-range studies have shown that Cxc is primarily capable of colonizing grass species. Recombinant strains of Cxc that are potential InCide candidates show host-colonization patterns very similar to the wild-type organism.

Extensive laboratory, greenhouse, and field studies have been conducted since 1986 to determine the ability of Cxc and/or Cxc/Bt to persist in the environment and disperse beyond the point of introduction (EUP58788-EUP1, 1987; -EUP2, 1989). Survival time of Cxc and a Cxc/Bt strain on the phylloplane of four plant species (corn, radish, marigold, and soybean) was shown to be a maximum of 2 weeks under greenhouse and growth chamber conditions. This characteristic can be attributed to the inability of these microbes to replicate outside a living host plant and the absence of a resting spore stage. Cxc is also short-lived in soil, water, and debris. For example, in 1987 field trials in Maryland, Cxc was undetectable after 2 weeks in soil, 3 weeks in soil-incorporated green residue of colonized corn plants, 5 weeks in buried sections of cornstalks and 7 weeks in cornstalks standing in the field after harvest.

Field studies conducted with a prototype recombinant Cxc (Cxc/Bt) in 1988 again demonstrated poor persistence in soil (1–5 weeks), incorporated plant material (6–8 weeks), and in cornstalks remaining after harvest (6–10 weeks). Cxc/Bt was not detected at all in soil around inoculated plants, even after incorporation of the plant material into the soil.

Results on persistence in 1989 in corn debris were similar to the 1988 data described above. Decline of Cxc and Cxc/Bt over time after harvest was rapid and complete. In 1987 and 1988 field experiments, the endophyte was never detected in runoff water from plots of colonized corn, before or after colonized plant debris was chopped and incorporated. This provides additional evidence that a colonized corn crop is not likely to provide a source of soil inoculum for subsequent crops.

Field trials in 1988 demonstrated that Cxc/Bt was not naturally dispersed from corn to other corn plants or weeds. In an attempt to induce artificial transmission, colonized corn plants were repeatedly cut with shears until the shears were wet with sap, and the shears were then used to trim noncolonized weeds; only in this case was any mechanical transmission observed, and even then the frequency of transmission was quite low. Cxc/Bt did not colonize any of the trap plants (corn and bermudagrass)

planted around the perimeter of the test sites used to monitor dispersal of the microbe. Plant-to-plant dispersal of Cxc from inoculum foci was rarely observed, and when it occurred, transmission was by mechanical means (i.e., cultivation designed to exaggerate normal farming practices).

There was no spread of recombinant Cxc/Bt to trap plants in the 1989 or 1990 field studies. These plots were replanted to bermudagrass for monitoring purposes in 1990 and no overwintering of the endophyte was observed.

20.3 Genetic Engineering of Cxc

Bacillus thuringiensis (Bt) is a Gram-positive bacterium that has been used commercially as a biological insecticide for more than three decades. Over 1,000 Bt strains and isolates have been identified, most of them active against larval Lepidoptera but exhibiting different ranges of activity within this group. This extreme specificity is responsible for the attractiveness of Bt as an environmentally safe insecticide which has little or no toxicity toward vertebrates or beneficial insects.

During sporulation, Bt cells produce a crystalline inclusion that is released with the spores upon cell lysis. Most commercial formulations of Bt, such as Dipel™, consist of a mixture of spores and crystals. Upon ingestion by a susceptible caterpillar, the proteinaceous crystal is degraded in the alkaline, reducing environment of the midgut, releasing a protoxin that can be digested by the insect's proteolytic enzymes (Fast, 1981). This process releases one or more delta endotoxins, the number and specificity of which depend on the Bt strain and the insecticidal crystal protein genes expressed (Hofte and Whiteley, 1989). An important step in toxicity appears to be the binding of toxin molecules to receptor sites on the brush border membrane of the midgut epithelium (Hoffman, 1988). Subsequent steps in the process are still under debate, but the end result is loss of integrity of the epithelial cells and consequent disintegration of the gut wall. The insect stops feeding and either dies immediately due to catastrophic increase in pH of the hemolymph (due to leakage of gut contents), starves to death, or succumbs to septicemia when opportunistic microflora (including Bt) invade the hemocoel from the gut (Fast, 1981).

Recombinant DNA techniques were used to modify wild-type Cxc to produce delta-endotoxin proteins of *Bacillus thuringiensis* subsp. *kurstaki* strain HD-73. The Cxc/Bt recombinant strains contain either: (1) the intact cryIA(c) protoxin gene of HD73 (coding for the 130-kDa protoxin that is broken down via proteolysis in the alkaline gut of the corn borer to form the activated toxin); (2) gene fusions combining the toxic domain of HD-73 with various marker genes; or (3) the toxic domain itself (coding for the active endotoxin). Molecular geneticists at CGI have constructed artificial plasmids that include: (1) the toxin coding region; (2) regulatory sequences that

control transcription of the genetic code to messenger RNA (promoters); and (3) marker genes that confer selectable traits (such as resistance to antibiotics) for detection of transformants. The common enteric bacterium *Escherichia coli* is used as a host for transformation with the cloning vectors for the initial construction of these expression cassettes. Successful cassettes are then cloned into proprietary integration vectors which contain a segment of DNA homologous to a segment of the chromosomal DNA of Cxc.

When the integration vector is inserted into the Cxc cell, crossing-over occurs between the homologous regions of the vector and the host chromosome, resulting in the stable insertion of the engineered DNA sequence into the Cxc chromosome. The resulting Cxc/Bt recombinant produces HD-73 toxin proteins that can be identified by Western blotting (Burnette, 1981) against crystal protein purified from sporulated HD-73 cultures.

Because the wild-type parental strain of Cxc/Bt has no detectable plasmids or prophage (which could mobilize recombinant genes in nature), and because Cxc isolates have proven unable to transmit or exchange integrated DNA sequences with other bacteria (EUP58788-EUP1, 1987), there is minimal risk of genetic exchange of recombinant toxin genes between Cxc/Bt and other microorganisms. Cxc/Bt recombinant strains have also been shown to segregate spontaneously, losing the engineered gene sequences at a low frequency. Segregants (which can be distinguished from recombinants by use of selective media) are able to out-compete recombinants, so that the recombinant genes are eventually lost from a Cxc population in the host plant (EUP58788-EUP2, 1989; -EUP4, 1990). This reversion phenomenon occurs at a rate slow enough to ensure product performance (i.e., sufficient Cxc/Bt populations) within a growing season, but rapidly enough so that toxin genes could not persist in the environment in the unlikely event that Cxc/Bt were transmitted by mechanical means to a non-crop host plant such as bermudagrass.

20.4 Effects of Cxc/Bt on European Corn Borer

Unlike Bt, Cxc/Bt does not sporulate and release crystal toxins, which can then be ingested by caterpillars. Cxc/Bt does not secrete its toxin, so that the entire bacterial cell must be digested by the insect in order to release its active ingredient. However, once this occurs, the symptoms are similar to those observed in larvae ingesting Bt: feeding slows and eventually stops, and larvae die from starvation or from invasion of the hemocoel by opportunistic microorganisms (Fast, 1981).

Experiments have demonstrated that Cxc/Bt can prevent or reduce European corn borer (ECB) damage to inoculated field corn under conditions of artificial infestation in both greenhouse and field. In greenhouse experiments (described in detail in EUP58788-EUP3, 1989), field corn seedlings were inoculated with Cxc approximately 2 weeks after planting (V3 to

V4 stage) by injection 1 to 2 cm above the soil line with 10^7 to 10^8 CFU in phosphate buffered saline. Plants that were confirmed to be Cxc-colonized by phase contrast microscopy of leaf midrib sap were infested 5 or 6 weeks after inoculation by placing neonate ECB larvae in holes drilled into the stalks (Chiang, 1959). Plants were dissected 3 to 4 weeks later to assess tunneling damage and number of surviving insects. Inoculation with the Cxc/Bt recombinant led to significant ($P \leq 0.05$) reduction in survival and damage. When averaged across three separate trials, Cxc/Bt-colonized plants contained 60% fewer live ECB larvae and tunnels, and total tunnel length per plant (cm) was reduced by 70%, relative to controls inoculated with wild-type Cxc or sterile buffer.

A fourth trial was infested by placing neonate larvae into leaf axils at or near pollen shed to simulate more realistically a natural infestation by second brood ECB (Showers et al., 1989). In this trial, the effect of Cxc/Bt inoculation on ECB parameters was less pronounced (35% to 50% reduction relative to controls), but was nevertheless statistically significant. A field test (described more fully in EUP58788-EUP4, 1990) was conducted in a manner similar to the fourth greenhouse trial (i.e., infested in leaf axils at pollen shed), using the same three inoculation treatments in six different corn hybrids, with similar results. Inoculation with Cxc/Bt caused significant overall reduction in borer survival and tunnel damage, although the magnitude and consistency of this effect varied between hybrids.

Current efforts are aimed at development of more potent strains of Cxc/Bt (producing more of the Bt toxin), elucidation of the relationship between Cxc/Bt population and Bt expression levels in plants and activity against ECB, and selection of corn hybrids which interact most favorably with Cxc/Bt colonization to produce the desired insect suppression and yield benefit.

20.5 Seed Inoculation Technology

Since 1986, CGI has developed proprietary methods for inoculation of Cxc/Bt into corn seeds for delivery to growers via seed company licensees. Early efforts focused on existing seed delivery systems. Various methods for external seed application were examined, including seed pelleting and coating with Cxc contained in a variety of polymers, oils, and powders. Both needle-less injectors and microparticle guns were used for direct injection into the seed. None of these methods appeared commercially useful for producing colonized plants.

The use of a pressure differential to force Cxc-containing suspensions into dry seeds was successful, although this method produced only low percentages of colonized plants and resulted in a precipitous drop in germination and seedling vigor. However, if seeds are subjected to a period of imbibition in water prior to pressure inoculation with Cxc (Figure

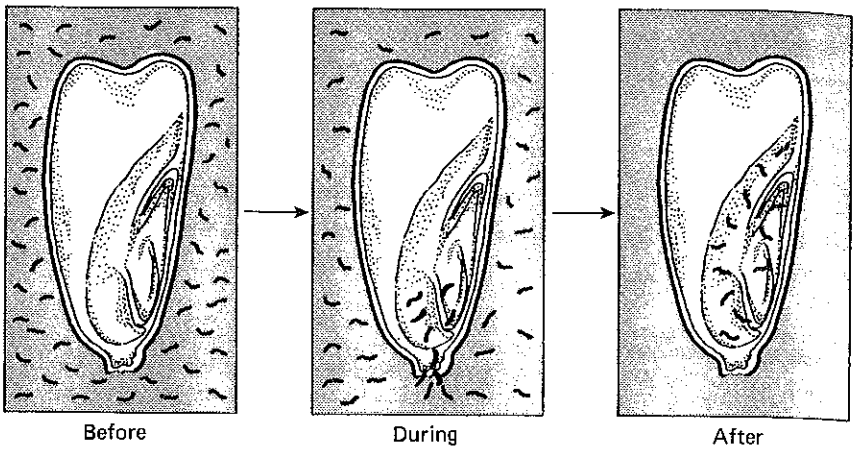
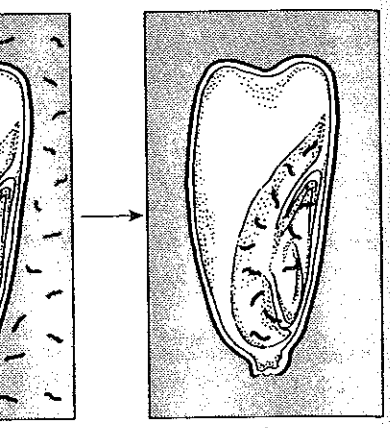


Figure 20.2 Seed inoculation with endophytic bacteria. Seeds are immersed in a buffered, high-density suspension of Cxc. A pressure differential is then applied, forcing the bacteria into the seed via the pedicel. Upon removal from the pressure chamber, bacteria remaining in the embryo lead to colonization of the resultant plant.

20.2), up to 100% of the seeds treated can produce vigorous, endophyte-colonized plants. The current inoculation protocol involves imbibition followed by application of a pressure differential in a vessel containing a buffered suspension of Cxc cells. Bacterial cells can be either harvested from petri plates or from a fermenter using standard procedures and SC or S8 media (Davis et al., 1980). Seeds are then removed from the inoculation suspension and dried on a forced air dryer after which conventional seed treatments can be applied (Figure 20.3). Field studies have demonstrated that a wide range of commercially important field and sweet corn hybrids can be successfully inoculated. Application of commonly used seed treatments (Captan™, NuGro™ Insecticide, and Color Coat Red™) appears to have no adverse impact on bacterial survival or efficacy of inoculation. Storage of inoculated seeds for longer than a year is possible, with only a gradual reduction in bacterial titer and no significant effect on seed germination. Although shelf life for crops is anticipated to vary, a shelf life of one season is expected for the first commercial corn seed product.

Development of an effective seed inoculation technology made larger-scale field trials possible in 1989. These trials were conducted in Maryland, Illinois, Minnesota, and Nebraska with plants from seed inoculated with Cxc and Cxc/Bt. Stand quality and seedling vigor were affected by the inoculation process in some environments, with some hybrids, but this inoculation effect disappeared as the season progressed. There were no apparent effects of the inoculation process at the time of harvest. Although there were yield reductions across test sites, which could be attributed to



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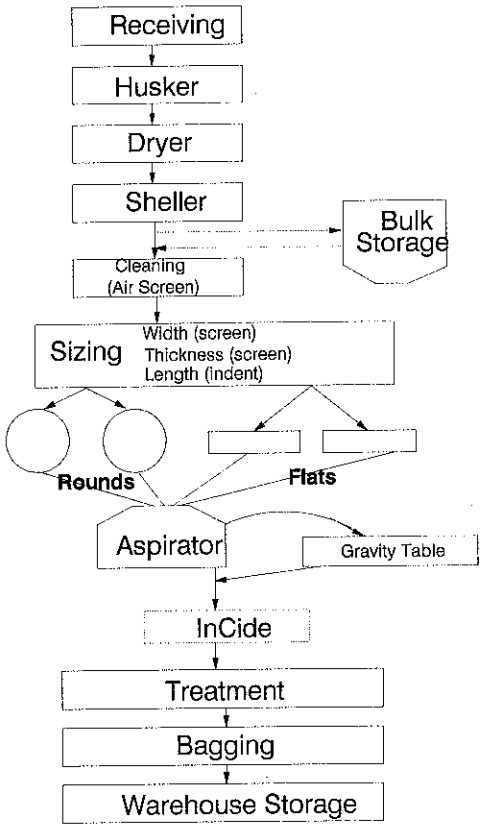

Figure 20.3 Integration of endophytic delivery to seeds into the operation of a seed conditioning plant. Bulk, harvested corn cobs are husked, dried, shelled, cleaned, and sized. InCide™ endophytes produced by classical fermentation will be added at this point in the process, residual added moisture will be removed, and seeds will reenter the conditioning train to be treated with prophylactic chemicals to prevent storage and seedling fungal and insect damage. Seeds are then bagged and stored.

the presence of the endophyte, certain hybrids performed well and will be candidate hybrids for initial product development.

Industrial-scale seed inoculation will be performed with custom-built machinery designed to be incorporated into the lines of current seed conditioning plants with only minimal need for redesign of existing facilities. CGI is currently working with four seed companies on the development and field testing of InCide technology. These companies are DeKalb Plant Genetics (DeKalb, Illinois), NC+ Hybrids (Lincoln, Nebraska), and Hoegemeyer Hybrids (Hooper, Nebraska), and Rogers Brothers Seed Co. (Boise, Idaho).

20.6 Conclusions and Future Directions

CGI expects that the following advantages of InCide biopesticide technology will make InCide products commercially successful compared to externally applied insecticides and fungicides: (1) *Environmental safety*:

Endophyte-based plant vaccines will be safe for farm workers, consumers and wildlife and will not pollute the environment. (2) *Farmer convenience*: Farmers will not need to spray insecticides or fungicides against targeted pests because endophyte-based vaccines will be applied to seeds by seed companies using CGI's proprietary technology. (3) *Minimum disturbance*: The use of InCide products will not require alteration of current agricultural practices. (4) *Consistent dosage*: Endophyte-based delivery systems will provide consistent, continuous levels of protection against the targeted pest. In contrast, externally applied pesticides can be either over-applied or under-applied. (5) *Season-long protection*: Endophyte vaccines will live within the vascular system for the life of the plant, where they are protected from abiotic factors. In contrast, externally applied products can be rendered ineffective by rain, sunlight, wind, and other environmental forces and often require multiple applications. (6) *Low-cost manufacturing*: Only a minute amount of the endophyte is required for each seed because the endophyte multiplies inside the growing plant. Externally applied pesticides require a large-scale manufacturing process.

Endophytes can be found throughout the plant kingdom. We are using the tools of biotechnology to add specific beneficial qualities to carefully chosen endophytes. By selective enhancement, endophytes can be engineered to help solve some of agriculture's most pressing problems.

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